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Research paper

Association of TLR polymorphisms with bronchopulmonary dysplasia

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ABSTRACT

Background: Bronchopulmonary dysplasia (BPD) remains a leading cause of morbidity and mortality during infancy. Evidence suggests that the Toll-like receptor (*TLR*) signaling pathway plays an integral role in lung inflammation and injury. This study aimed to detect single nucleotide polymorphisms (SNPs) in TLR pathway genes [*TLR5* and Toll-interleukin 1 receptor domain-containing adaptor protein (*TIRAP*)] among preterm neonates and to determine their association with the development and severity of bronchopulmonary dysplasia.

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Patients and methods

This cohort study included 101 preterm neonates admitted to the neonatal intensive care unit. Fifty preterm neonates developed bronchopulmonary dysplasia, and 51 did not who were considered as controls. *TIRAP*(rs8177374) polymorphism was genotyped by PCR-RFLP technique and *TLR5* (rs5744168) polymorphism by allele-specific polymerase chain reaction.

Results

The distribution of the *TLR 5* and *TIRAP* genotypes did not differ significantly between patients and controls. However, our results suggest that the presence of at least one copy of the *TIRAP* (2054C>T) variant may be associated with severity of BPD among preterm neonates.

By logistic regression analysis, duration of CPAP therapy and duration of mechanical ventilation were the significant independent variables associated with the development of BPD.

Conclusion

Our data support a potential role of *TIRAP* (2054C>T) polymorphism on the severity of BPD among preterm neonates. Further studies are warranted to confirm these findings, and to further study the interactions between clinical and genetic risk factors of BPD.

1. Introduction

The Toll-like receptor (*TLR*) signaling pathway proteins are pattern recognition receptors that recognize signature microbial motifs and stress ligands (Akira et al., 2006). TLR-mediated innate immune responses play an important role in preventing bacterial invasion, maintaining mucosal homeostasis and regulating inflammation (Akira et al., 2006; Rakoff-Nahoum et al., 2004).

Most, but not all, TLR proteins signal through a complex intracellular cascade utilizing the *Myeloid differentiation primary response gene 88 (MYD88)* pathway. TLRs function as an inflammation switch and consequently are of interest in the study of neonatal inflammatory conditions (Petrikin et al., 2010). They have been implicated in various conditions affecting newborns, including necrotizing enterocolitis (Fusunyan et al., 2001), bronchopulmonary dysplasia (Basu and Fenton, 2004;

Abbreviations: BPD, Bronchopulmonary dysplasia; TIRAP, Toll-interleukin 1 receptor domain-containing adaptor protein; SNPs, Single nucleotide polymorphisms; TLRs, Toll-like receptors; NICU, Neonatal intensive care unit; NICHD, The National Institute of Child Health and Human Development; PCR-RFLP, Polymerase chain reaction- restriction fragment length polymorphisms; EDTA, Ethylenediaminetetra-acetic acid; DNA, Deoxyribonucleic acid; SD, Standard deviation; MBL, Mannose binding lectin; MMP-16, Matrix metalloproteinase 16; VEGF, Vascular endothelial growth factor; SPOCK2, Sparc/Osteonectin, CWCV, and Kazal-like domains proteoglycan; IL, Interleukin; PRBC, packed red blood cell; IL-18R1, Interleukin-18 receptor 1; IL18RAP, Interleukin 18 receptor accessory protein; SP-A, Surfactant protein A; SP-B, Surfactant protein B; SP-C, Surfactant protein C.

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Chaudhuri et al., 2007; Raymond et al., 2007)), preterm birth (Krediet et al., 2007), inflammatory bowel disease (Abreu and Arditi, 2004), differential response to bacterial sepsis (Sadeghi et al., 2007; Viemann et al., 2005), and as mediators of the sequelae of maternal smoking (Noakes et al., 2006).

TLR5 is involved in the recognition of flagellin protein component of bacterial flagella. Ligation of these innate immune receptors, which are poised on the surface or organellar membranes of many cell types, including epithelial cells, macrophages and dendritic cells, initiates signaling cascades that trigger secretion of inflammatory cytokines and chemokines as well as expression of co-stimulatory signals for adaptive immunity (Ivison et al., 2007). TLR5 is thought to transduce signals via the MyD88-dependent pathway only (Sierro et al., 2001; Steiner et al., 2000).

Toll-interleukin 1 receptor domain-containing adaptor protein (*TIRAP*) is an adapter in the TLR pathway that plays a key role in MyD88-dependent *TLR2* and *TLR4* responses (Akira et al., 2006). The partial loss of function arising from this variant may attenuate immune responses to both Gram-positive and Gram-negative bacteria (Khor et al., 2007).

Sepsis and the systemic inflammatory response increase the likelihood of BPD in premature infants. The inflammatory response in the lung results in the production of pro-inflammatory cytokines, migration of PMNs and changes in vascular permeability (Wynn et al., 2010; Cornell et al., 2010). The presence of these factors likely causes immediate damage to alveoli and capillaries, but also may be related to the long-term arrest of alveolarization seen in infants with BPD (Trembath and Laughon, 2012).

Emerging evidence suggests that the TLR signaling pathway is a key component of a pulmonary homeostatic program that abrogates lung inflammation and injury (Sampath et al., 2012). So far, there are little human researches about association with neonatal diseases and TLRs.

The aim of our study was to detect SNPs in Toll-like receptor pathway genes [*TLR5 and TIRAP*] among preterm neonates and to determine their association with the development and severity of bronchopulmonary dysplasia.

2. Patients and methods

One hundred and one preterm neonates were recruited prospectively from the neonatal intensive care unit (NICU) of Cairo University Hospital throughout the period from April to December 2014. Neonates who were delivered prematurely and admitted to the NICU were enrolled. Neonates who required oxygen therapy >21% for at least 28 days comprised the patient group (50), and preterm neonates who did not require >21% oxygen therapy for the first 28 days of life were considered as controls (51).

The National Institute of Child Health and Human Development (NICHD) severity-based definition of BPD was used (Table 1) (Jobe and Bancalari, 2001).

Prenatal and postnatal risk factors of BPD, complications during admission and outcome were recorded. Necrotizing enterocolitis was diagnosed on the basis of clinical and radiological modified Bell staging criteria (Bell et al., 1978). Intraventricular hemorrhage (IVH) was diagnosed by real-time portable cranial ultrasound (Volpe, 2008). The presence of a Patent ductus arteriosus (PDA) was diagnosed by echocardiography. Sepsis was diagnosed by a positive blood culture, or a positive C-reactive protein (CRP) and immature to total neutrophil (I:T) ratio >2 in the presence of clinical signs of sepsis if blood culture results were not available. Early-onset sepsis was defined as occurring in the first 3 postnatal days, while late-onset sepsis (LOS) was defined as occurring at >72 h of life. Infants with major congenital anomalies were excluded.

Informed consents were obtained from parents of studied subjects. The study design was approved by the Scientific Research Committee of the Pediatric Department, Faculty of Medicine, Cairo University. Data confidentiality was observed according to the Revised Helsinki Declaration of Bioethics (World Medical Association Declaration of Helsinki, 2013).

Laboratory Procedure: Study of selected SNPs in the TLR pathway (targeting *TLR5, TIRAP* genes). *TIRAP* (2054C>T) polymorphism was genotyped by PCR-RFLP technique and *TLR5* polymorphism by allele-specific polymerase chain reaction.

2.1. TIRAP (rs8177374) (2054C>T) genotyping by PCR-RFLP technique

2 mL blood were withdrawn aseptically from every patient and control then collected in sterile ethylenediaminetetra-acetic acid (EDTA) vacutainer tube. DNA was extracted from the whole blood using DNA extraction kit (GeneJET™ Genomic DNA Purification Kit, Fermentas LIFE SCIENCE, catalog number: #K0721, Vilnius, Lithuania). *TIRAP* (2054C>T) genotyping was determined as described by Castiblanco et al., 2008. The primers (Fermentas™–Lithuania) were forwards (5′-CTC CAG GGG CCG AGG GCT GCA CCA TCC CCA TGC TG -3′) and reverse (5′-TAC TGT AGC TGA ATC CCG TTC C-3′) (Castiblanco et al., 2008). All reactions were performed in a total volume of 25 μL. After an initial denaturation step (4 min at 95 °C), the samples were subjected to 35 cycles of 95 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s, with a final extension of 5 min at 72 °C. The PCR products were digested with Thermo Scientific FastDigest *Bst*XI Lot (#FD1024) (Thermo Fisher Scientific Inc., USA), and the restriction fragments were analyzed on a 2% agarose gel. In the case of wild (CC) genotype, a band at 191 bp was observed. In the presence of heterozygosity (CT), two bands (191 bp and 164 bp) were distinguished. When an individual is homozygous for the mutant-type (TT), only one small fragment of 164 bp is detectable in the gel.

2.2. TLR5 (rs5744168) (1174C>T) polymorphism genotyping by allele-specific polymerase chain reaction (AS-PCR) technique

Primers were designed that bound either to the wild-type sequence beginning with C at position 1174 (5′ TTACAGACCTTGGATCTCC-3′) or to the mutant sequence beginning with T at position 1174 (5′-TTACAG ACCTTGGATCTCT-3′). The polymorphism-specific primers were used together with a conserved reverse primer (5′-CAGAATCTGGAGATGA GGTACCCG-3′).

and internal control primers specific for human growth hormone (5′ CAGTGCCTTCCCAACCATTCCCTTA-3′ [forward] and 5′- ATCCACTCACGG ATTTCTGTTGTGTTTC-3′ [reverse]) (Dunstan et al., 2005).

Two PCRs that used either the C-specific or the T-specific primer together with the reverse primer (203-bp product) and the internal control primers (480-bp product) were performed on each DNA sample. Standard PCR cycling conditions were used, with an annealing

Table 1
NICHD severity-based definition of BPD.

Gestational age at birth	Mild BPD	Moderate BPD	Severe BPD
<32 week	Room air at 36 week PMA or discharge*	<30% oxygen at 36 week PMA or discharge*	≥30% oxygen and/or positive pressure at 36 week PMA or discharge*
≥32 week	Room air by 56 d postnatal age or discharge*	<30% oxygen at 56 d postnatal age or discharge*	≥30% oxygen and/or positive pressure at 56 d postnatal age or discharge*

PMA: Post menstrual age. *All categories require treatment with >21% oxygen for at least 28 days, then assessment at PMA/postnatal age or discharge whichever comes first.

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